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Effect of temperature regime on diapause intensity in an adult-wintering Hymenopteran with obligate diapause

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ABSTRACT

Osmia lignaria is a solitary bee that over-winters as a fully eclosed, cocooned, unfed adult. Our objective is to understand the effect of wintering temperature on diapause maintenance and termination in this species. We measure respiration rates and weight loss in individuals exposed to various wintering temperatures (0, 4, 7, 22 °C, outdoors) and durations (28, 84, 140, 196, 252 days). We use time to emerge and respiration response (respiration rate measured at 22 °C) as indicators of diapause intensity. Adults spontaneously lower their respiration rates to ~0.1 ml/g h within 1 month after adult eclosion, indicating obligatory diapause. Non-wintered individuals maintain low respiration rates, but lose weight rapidly and die by mid-winter. In wintered adults, two phases can be distinguished. First, respiration response undergoes a rapid increase and then reaches a plateau. This phase is similar in bees wintered at 0, 4 and 7 °C. In the second phase, respiration response undergoes an exponential increase, which is more pronounced at the warmer temperatures. Composite exponential functions provide a good fit to the observed respiration patterns. Adults whose respiration response has reached 0.45 ml/g h emerge promptly when exposed to 20 °C, indicating diapause completion. Individuals wintered for short periods do not reach such respiration levels. When exposed to 20 °C these individuals lower their metabolic rate, and their emergence time is extended. The relationship between respiration rates and emergence time follows a negative exponential function. We propose two alternative models of diapause termination to interpret these results.

1. Introduction

Diapause is the primary mechanism whereby insects of temperate zones synchronize their life cycle with seasonal changes. Diapause may be succinctly defined as a genetically programmed, neurohormonally mediated, dynamic state of low metabolic activity during which morphogenesis ceases or significantly slows down (Tauber et al., 1986; Danks, 1987). To emphasize diapause as a *process* rather than as a *status*, Andrewartha (1952) coined the term *diapause development*. However the use of the word "development" to describe a process of "arrest of development" has caused some confusion (Hodek, 2002), and we thus use the more intuitive terms *diapause initiation*, *maintenance* and *termination* (Kostal, 2006). In principle, the physiological differences between these phases are clear, but researchers do not always agree on how to characterize their transitions, especially as it regards to diapause termination (Kostal, 2006). This is so for several reasons. First, diapause does not entail a complete cessation of development (Hahn and Denlinger, 2007). Response to environmental factors such as temperature, photoperiod and humidity, are far from constant during the course of diapause (Gray et al., 1995; Kostal, 2006) and even at the lowest metabolic rates some degree of biochemical activity occurs (Danks, 1987). Although morphogenesis is arrested, growth, mobility and feeding may sometimes take place during diapause (Hodek, 2002; Kostal et al., 2008). Second, the physiological mechanisms underlying diapause are highly variable. This is not surprising, given that diapause has appeared many times independently during the course of arthropod evolution (Tauber et al., 1986), and may occur at different developmental stages, even among closely related species (Tauber et al., 1986; Danks, 1987). This variability makes it difficult to establish general principles, and to apply results from one taxon to another. For example, respiration rates during diapause vary widely between species, both in absolute terms and in relation to respiration rates during non-diapause (Table 5 in Danks, 1987). Similarly, chilling (exposure to "cold" temperatures)

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is a prerequisite for diapause development in some species but not in others (Hodek and Hodková, 1988; Tables 2 and 3 in Hodek, 2002). Third, it is difficult to extrapolate results obtained in the laboratory (with a clear demarcation of environmental factors), to field conditions, with environmental factors changing and fluctuating in complex ways (Hodek, 2002; Kostal, 2006). It is not always clear whether observed physiological responses are attributable to diapause termination or to the experimental manipulations themselves (Ragland et al., 2009). Many temperate-zone insects are known to terminate diapause by mid-winter, and then remain in a state of post-diapause quiescence during which the insect has the potential to resume morphogenesis, which at that point is inhibited by low temperature (Tauber et al., 1986; Table 1 in Hodek, 2002). The insect is then able to respond immediately to development- or activation-promoting conditions. For this reason, it is usually assumed that diapause under field conditions is completed 4–5 months after its initiation (Hodek, 2002). However, there is some debate as to whether the transition between diapause and post-diapause quiescence is gradual or abrupt (Sawyer et al., 1993; Gray et al., 1995).

In this study we investigate the effect of temperature on diapause initiation, maintenance and termination in the solitary bee *Osmia lignaria* (Hymenoptera, Megachilidae). This species shows several biological traits that make it an interesting model for a study of this sort. First, in contrast to most other species in which diapause has been studied, *O. lignaria* is an obligate diapauser. Second, development from egg to adult in this species takes place inside a sealed nest in complete darkness. Therefore, as opposed to other diapause models (Tables 21 and 22 in Danks, 1987; Kostal et al., 2000, 2008), photoperiod does not play a key role in diapause maintenance or termination in *O. lignaria*. Third, *O. lignaria* over-winter as fully eclosed adults within their natal cocoon and therefore have no access to food during pre-wintering. This is in contrast to most other adult-wintering insects whose diapause has been studied (including a bumblebee, *Bombus terrestris*), in which pre-wintering adults ingest food and build up their metabolic reserves in preparation for winter (Tauber et al., 1986; Hodková and Hodek, 1989; Hodek and Honek, 1996; Beekman et al., 1998).

Most studies use the time required for overt resumption of development or reproduction when exposed to diapause-terminating conditions as a measure of diapause intensity and termination (Kostal, 2006). Fewer studies (e.g., Gray et al., 1995; Yaginuma and Yamashita, 1999; Singtripop et al., 2007; Ragland et al., 2009) use respiration rates. In this study we measure respiration rates and time required to emerge in *O. lignaria* females exposed to various wintering temperatures, including field temperatures, and various wintering durations. Our objective is to understand the effect of temperature regime on winter diapause initiation, maintenance and termination in this species. *O. lignaria* reaches the adult stage towards the end of summer. Then, until the onset of winter, fully formed adults within their cocoons undergo a period during which temperatures are still appropriate for development (pre-wintering period). Previous studies (Kemp et al., 2004; Bosch et al., 2008) have shown that during this period adults lower their respiration rates from 0.20 to 0.25 ml/g h in the newly eclosed adult to 0.1 ml/g h. We address the following questions: (1) given that pre-wintering adults have already lowered their respiration rates, what is their respiratory response to chilling temperatures? (2) Can diapause be completed without chilling? (3) How does diapause maintenance proceed at different wintering temperatures? How do adults wintered for short periods respond to warm (incubation) temperatures? (4) Is it possible to establish a clear transition between diapause and post-diapause? (5) Can we use respiration rates during winter as a predictor of emergence time in the spring?

The answers to these questions are interpreted in the light of results on survival, vigour and emergence time obtained in a previous study in which similar temperature regimes were used (Bosch and Kemp, 2003).

2. Materials and methods

2.1. Life cycle of *O. lignaria*

O. lignaria is a spring-flying, solitary bee native to North America. Adults fly for about a month in early spring (March–April), during which time females build nests in pre-established cavities (typically, abandoned beetle burrows in dead wood). These nests are provisioned with pollen mixed with nectar as food for the progeny. Eggs hatch within 1–2 weeks, and larval development takes place through five instars. By early summer, the last instar finishes up the pollen-nectar provision, spins a thick cocoon and enters a short summer (prepupal) diapause (Kemp et al., 2004; Kemp and Bosch, 2005; Bosch et al., 2008). Pupation takes place 1–2 months after cocoon spinning, and adult eclosion occurs by late summer or early autumn (Bosch and Kemp, 2000). Fully eclosed adults harden their cuticle for about a day, but do not emerge out of the cocoon. They enter diapause in autumn (Kemp et al., 2004; Bosch et al., 2008), only to emerge in the following spring. Adult-wintering appears to be a derived state within the Megachilidae, most of which winter in the prepupal stage (Bosch et al., 2001). Wintering in the prepupal stage is also the prevalent state in most bees (Apiformes) and other Hymenoptera (Stephen et al., 1969; Gauld and Bolton, 1988). *O. lignaria* is an excellent pollinator of fruit tree flowers. For this reason, management methods have been developed to use populations of this species for commercial orchard pollination (Torchio, 1985; Bosch and Kemp, 2002).

2.2. Experiment 1: effect of wintering temperatures

2.2.1. Populations and rearing methods

We used the progeny of an *O. lignaria* population released in early May 2002 in an apple orchard in North Logan, Utah, USA. Drilled wood blocks with inserted paper straws (length: 15 cm; diameter: 7.5 mm) were used as nesting materials. By the end of the nesting period (20 June), some straws in which nests had been built were pulled out of the wood blocks and brought to the laboratory, where they were kept in a 22 °C cabinet. Other nests were reinserted in wood blocks and stored in a North-facing open barn in the same apple orchard. Temperature in the barn was recorded hourly with a temperature logger. Beginning on 5 August, when bees started to reach adulthood, we X-rayed all nests (Stephen and Undurraga, 1976) every 3 days. We used X-ray plates to sex individuals (females are usually larger than males and are located in the innermost cells within a nest; Torchio, 1989; Bosch and Kemp, 2001), and to establish adult eclosion dates.

From the group of nests kept at 22 °C, we selected 28 females (7 per treatment) that reached adulthood in mid August. These females (within their cocoons) were extracted from paper straws and placed individually in clear gel capsules and pre-wintered at 22 °C for 30 days, then acclimatized for 7 days at 14 °C, and finally transferred to wintering cabinets at 0, 4, and 7 °C, respectively. These bees were held at their respective winter temperatures until May or until individuals started emerging. In a fourth treatment, intended to test the effect of absence of wintering, bees were left at 22 °C throughout pre-wintering and wintering. An additional group of 7 females were selected from the nests kept in the orchard. These females were also placed individually in clear gel capsules and then kept within a ventilated plastic food container within the barn in the apple orchard (outdoors treatment). Temperature was measured with a data logger adjacent to the wintered cocoons.

2.2.2. Respiration rates and weight loss

The first respiration measurements were taken in mid September, 1 month after adult eclosion, when respiration rates had reached their minimum values (Sgolastra, 2007; Bosch et al., 2008). From then on, measurements were taken once a week until 1 October, and then once every 2 weeks until emergence or until 12 May.

Oxygen consumption and CO_2 production were measured using constant volume respirometry. We used a Sable Systems FC-1 O_2 Analyzer® and a Li-Cor CO_2 Analyzer® operating in differential mode with a 100 ml/min flow rate (http://www.sablesys.com/index.php). This allowed accuracy of measurement that exceeded 0.001% in detecting departures from an undepleted air stream that had been scrubbed of CO_2 and water vapour with a Drierite® - Ascarite® column. At each sample date, we measured the O_2 consumed and CO_2 produced by each of 7 individual bees for 2 h at 22 °C in a Peltier cabinet. Data were collected via the Sable Systems data acquisition program DATACAN® following manufacturer's protocols. Upon completion of a respirometry session, individual bees were weighed. For comparison purposes, O_2 and CO_2 levels were adjusted for the weight of each individual and expressed as ml/g h. The ratio between CO_2 production and O_2 consumption (respiratory quotient, RQ) was calculated for each respiration measurement. Weight loss was calculated as the percentage of fresh body weight after each respiration measurement in reference to the initial body weight measured at the beginning of wintering.

It is important to note that respiration measurements were conducted at 22 °C for all treatments. Thus, we did not measure actual respiration rates during diapause, but the metabolic response of diapausing bees (wintered at different temperatures) when exposed to 22 °C (the temperature at which respiration measurements were conducted). We therefore use the term "respiration response" throughout our study, and use the magnitude of this response as a measure of diapause intensity. This approach was chosen because we knew from previous studies that even in populations wintered for long periods, incubation at ~20 °C was required for female emergence (Bosch and Kemp, 2001; unpublished data), and we were interested in exploring the relationship between a measurable metabolic response and time to emerge following incubation (objective 5). We believe the short (2 h) exposure of bees to 22 °C during respiration measurements did not significantly affect the general course of diapause because our results are consistent with those obtained in a previous study in which new individuals were used in each respiration measure (Kemp et al., 2004).

2.2.3. Statistical analysis

We used repeated-measures ANOVA to analyze differences in weight loss (arcsine-transformed) and RQ among treatments (excluding the 22 °C and outdoors treatments) throughout the wintering period.

Visual inspection of the respiration data of treatments 0, 4 and 7 °C, showed an initial increase followed by a plateau, and then a second increase. We fitted a five-parameter composite function to describe the observed pattern:

$$R = (a_1 - a_2 \cdot e^{-a_3 \cdot t}) + a_4 \cdot e^{a_5 \cdot t}$$

where R is the respiration rate, t is the number of wintering days and a_1 to a_5 are fitted parameters. The first term of the equation describes the first phase as an inverted exponential rapidly reaching a plateau, and the second term describes the second exponential increase. The variance of O_2 and CO_2 data increased with time, especially at $t > 100$ days. For this reason, we log-transformed the data, which not only increased homoscedasticity but also markedly improved the convergence of the regression procedure.

2.3. Experiment 2: effect of wintering duration

2.3.1. Population and rearing methods

We used the progeny of an *O. lignaria* population released in a cherry orchard in North Ogden, Utah, USA, in April 2004. Nesting materials were similar to those used in experiment 1. Nests obtained were brought to the laboratory in early May, and kept in a temperature cabinet simulating Logan (50 Km from North Ogden) daily temperatures with a 12:12 h thermoperiod (May = 18:11 °C; June = 21:15 °C; July = 24:18 °C; August = 22:15 °C). The high temperature of each monthly thermoperiod was obtained as the average between the maximum and the mean monthly temperatures of the month. Similarly, the low temperature of each monthly thermoperiod was obtained as the average between the minimum and the mean monthly temperatures of each month.

As in experiment 1, nests were X-rayed every 3 days to monitor individual adult eclosion. On 23 August, approximately in the middle of the adult eclosion period, 50 newly eclosed females (within their cocoons) were selected. These females were placed in clear gel capsules and distributed among 5 wintering duration treatments: 28, 84, 140, 196 and 252 days. Bees of all treatments were pre-wintered at 22:15 °C (12:12 h) for 2 weeks, then acclimatized to 10 °C for 1 week, and finally wintered at 4 °C. Upon completion of each wintering treatment, adults within their cocoons were placed individually in glass vials and incubated at 20 °C. Cocoons were monitored daily for emergence. For each treatment, we calculated percent survival (individuals emerging completely out of the cocoon) and emergence time (interval between incubation date and emergence date).

2.3.2. Respiration rates and weight loss

Respiration rates and weight were measured on 7 females per treatment, following the same protocol as in experiment 1. Respiration measurements were taken at least at five selected intervals: the day after adult eclosion, at the end of the pre-wintering, 7 days after wintering (4 °C) initiation; and the day before and the day after beginning of incubation (20 °C). From then on, respiration rates were measured every 2 weeks until emergence. As in experiment 1, RQs were calculated for each respiration measurement. Weight loss during wintering was calculated as the difference between the weight at adult eclosion and at the end of wintering. Weight loss during incubation was calculated as the difference between weight at the end of wintering and at the last respiration measurement before emergence (or death in females not emerging).

2.3.3. Statistical analysis

Percent weight loss (arcsine-transformed) across wintering duration treatments was analyzed using one way-ANOVA. One way-ANOVA was also used to analyze the effect of wintering duration on emergence time (time between incubation and emergence) and RQ. One of our objectives was to predict emergence time as a function of respiration rates during wintering. Thus, we fit negative exponential models to describe the relationship between respiration rates at the end of wintering and incubation time required for emergence.

3. Results

3.1. Experiment 1: wintering temperature

3.1.1. Respiration rates

Respirometry measurements started 1 month after adult eclosion, when respiration rates were at minimum values (about 0.10 ml/g h). Following transfer from 14 °C to wintering temperatures (0, 4 and 7 °C, respectively), bees responded with a rapid

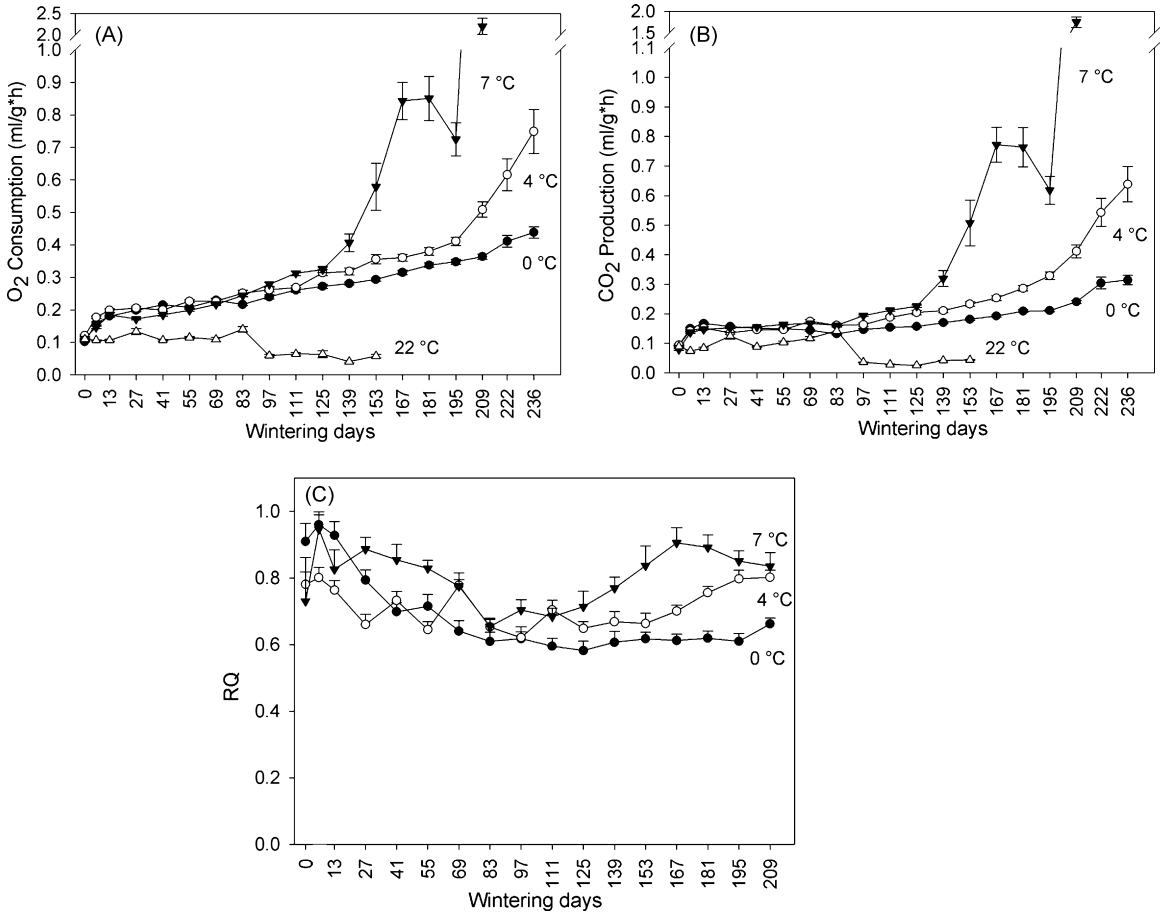

Fig. 1. Mean ± SE O_2 consumption (A), CO_2 production (B) and respiratory quotient (C) in *O. lignaria* females wintered at 0, 4, 7, and 22 °C. The first measurements were taken 1 month after adult eclosion, when respiration rates are at their lowest.

increase of their respiration response (Fig. 1). Within 2 weeks, respiration response reached a plateau during which CO_2 production remained approximately stable and O_2 consumption increased slowly. During this period, diapause intensity was similar in bees wintered at 0, 4 and 7 °C (Fig. 1). Then, in mid December (~100 days of wintering) respiration responses started to diverge among treatments, showing an exponential increase that was more pronounced (greater a_5 parameter in our non-linear model) at the warmer temperatures (Fig. 1). By mid April, bees of the 7 °C treatment started to emerge during the respiration measurement at 22 °C. Our model provided a good fit to the described relationship between respiration response and wintering days (Fig. 2, Table 1). RQ values first decreased, reaching minimum values towards mid-winter, and then increased in late-winter (Fig. 1; $F_{(16,288)} = 13.68$; $P < 0.0001$). RQ values differed among temperature treatments ($F_{(2,18)} = 13.52$; $P < 0.001$), with a significant wintering duration–temperature interaction ($F_{(32,288)} = 5.23$; $P < 0.0001$).

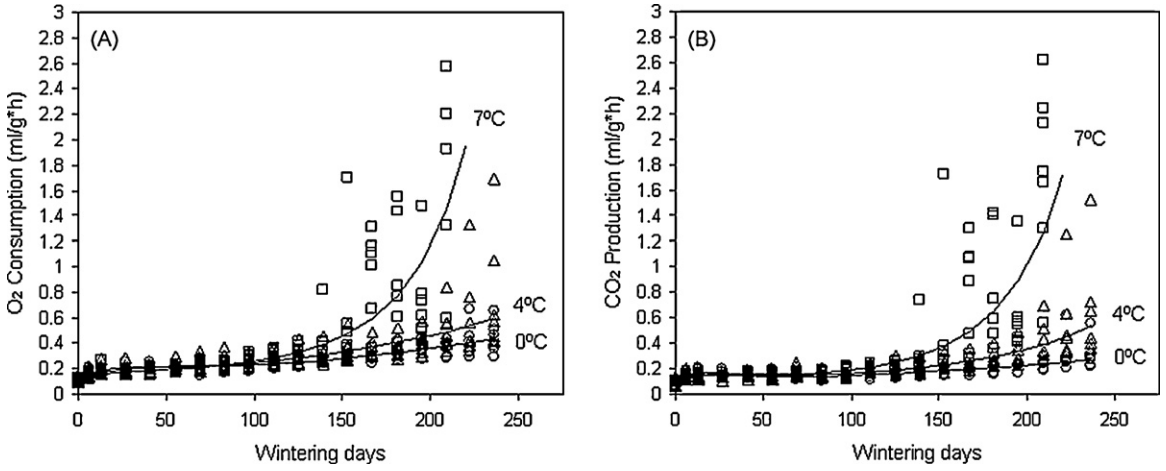

Fig. 2. Fitted non-linear regression of O_2 consumption (A) and CO_2 production (B) in *O. lignaria* females wintered at 0 (circles), 4 (triangles), and 7 °C (squares). Goodness-of-fit parameters are shown in Table 1.

Table 1
Goodness-of-fit parameters of the non-linear regression models of Fig. 2.

Temperature	R^2	F	P
O_2 consumption			
0 °C	80.1	1964.4	<0.0001
4 °C	74.6	781.1	<0.0001
7 °C	78.8	332.5	<0.0001
CO_2 production			
0 °C	68.3	2623.2	<0.0001
4 °C	76.1	1067.1	<0.0001
7 °C	80.8	444.2	<0.0001

The respiration response of bees wintered outdoors is shown in Fig. 3. As for bees wintered at 0, 4 and 7 °C, outdoors bees also increased their respiration response coinciding with an important temperature drop in early September, and then reached a plateau with a very slow increase. This plateau was longer than in the 0, 4 and 7 °C treatments, and then in early April, as soon as ambient temperatures reached 20 °C, respiration response skyrocketed and bees started to emerge. RQ levels in bees wintered outdoors followed a pattern similar to bees reared under artificial conditions reaching minimum values (~0.7) from December to February.

Bees of the 22 °C treatment were never exposed to cold temperatures. These bees never expressed the initial increase in respiration rates observed in the other treatments. Thus, respiration rates of bees kept at 22 °C remained low throughout the winter (Fig. 1).

3.1.2. Weight loss

The low respiration rates expressed by bees of the 22 °C treatment did not prevent them from loosing weight dramatically (Fig. 4). Within 3 months these bees had lost over 50% of their weight, and eventually all of them died within their cocoons. Bees

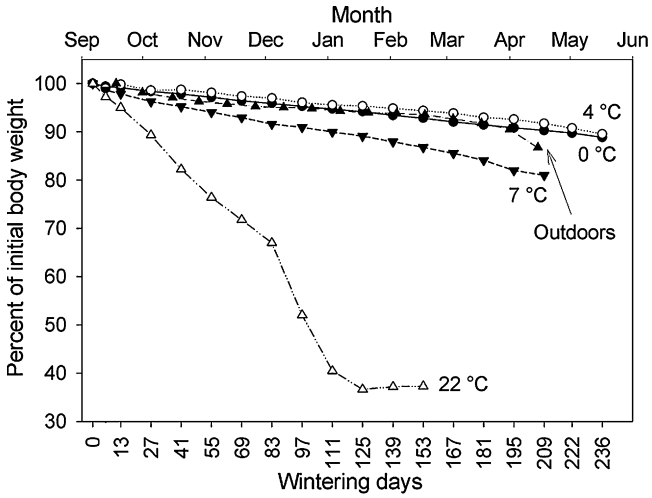

Fig. 4. Mean percent of initial body weight in *O. lignaria* females wintered at 0, 4, 7, 22 °C and outdoors. Error bars not shown for clarity.

of the other treatments also lost weight throughout the winter ($F_{(15,270)} = 863.64$; $P < 0.0001$), but at a much lower rate. Bees wintered at 7 °C lost significantly more weight (0.18 mg/day) than those wintered at 0 and 4 °C (0.06–0.07 mg/day; $F_{(2,18)} = 31.59$; $P < 0.0001$), and there was a significant interaction between temperature and wintering days ($F_{(30,270)} = 16.24$; $P < 0.0001$).

3.2. Experiment 2: wintering duration

3.2.1. Respiration rates

O_2 consumption and CO_2 production of newly eclosed adults were close to 0.25 ml/g h (Fig. 5). During pre-wintering (14 days at

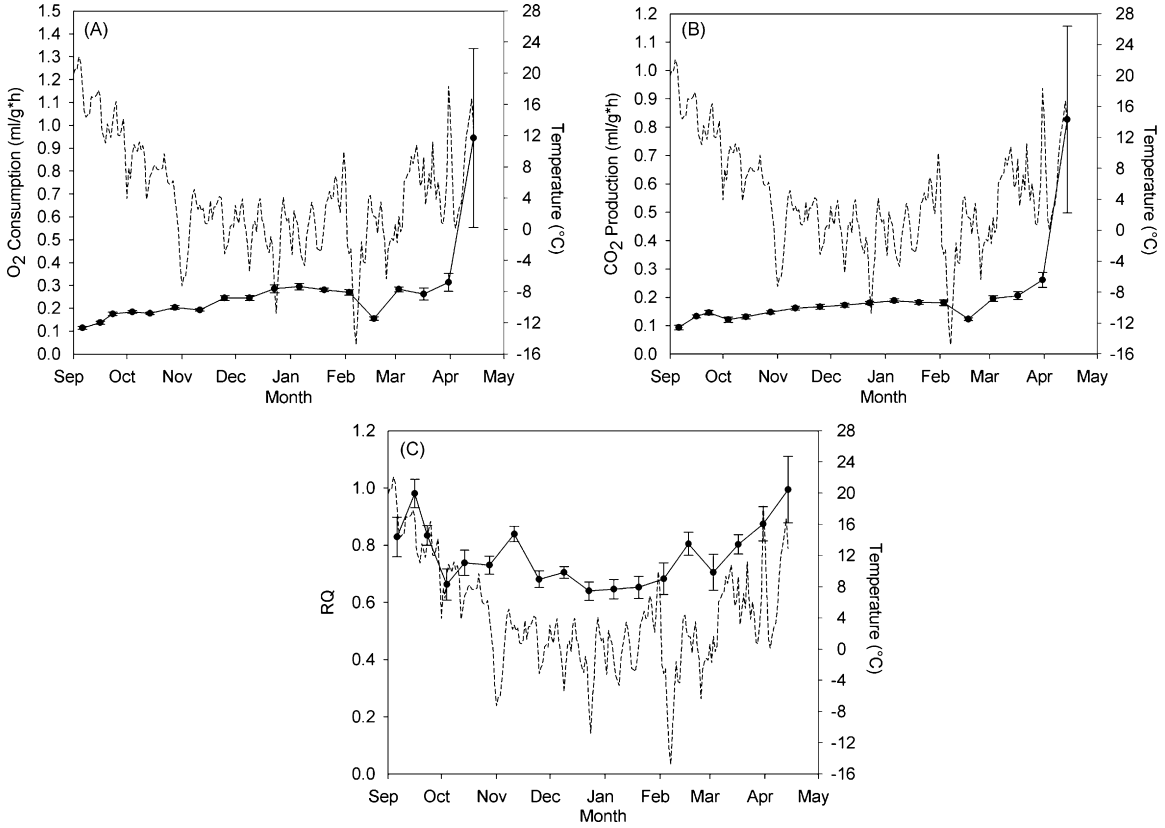

Fig. 3. Mean ± SE O_2 consumption (A), CO_2 production (B) and respiratory quotient (C) of *O. lignaria* females wintered outdoors. The first measurements were taken 1 month after adult eclosion, when respiration rates are at their lowest. Dotted line indicates mean daily ambient temperature.

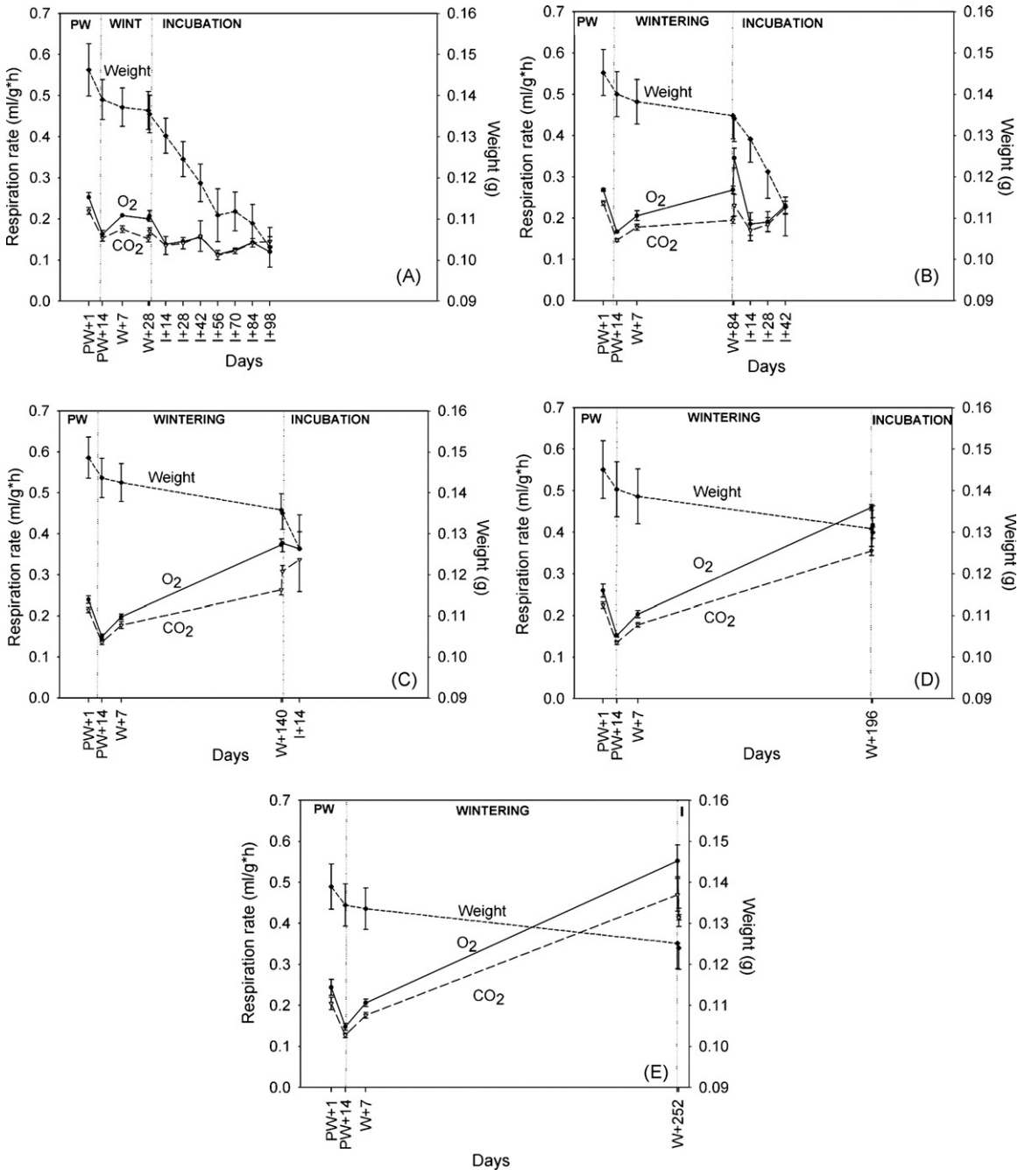

Fig. 5. Mean ± SE O_2 consumption, CO_2 production and weight during pre-wintering (PW), wintering (W) and incubation (I) in *O. lignaria* females wintered for 28 (A), 84 (B), 140 (C), 196 (D) and 252 (E) days at 4 °C. The first measurements were taken on the day of adult eclosion.

22–15 °C, 12:12 h), respiration response declined to ~0.15 ml/g h. As in experiment 1, as soon as bees were transferred to 10 °C, respiration response initiated a logistic climb and then continued to increase throughout wintering at a much slower rate. In treatments with short wintering durations (28, 84 days), bees were incubated at 20 °C while respiration response was still low. Upon incubation, females of these two treatments showed a tendency to increase their metabolic rates, but then rapidly lowered their O_2 consumption and CO_2 production to levels similar to those expressed during wintering (Fig. 5). Instead, bees wintered for longer periods (approaching natural wintering duration in northern Utah) did not lower their respiration rates during incubation (Fig. 5). RQ values followed a similar pattern to experiment 1, reaching minimum values towards mid-winter (84–140 days) (Fig. 6; $F_{(6,40)} = 6.52$; $P < 0.001$).

3.2.2. Weight loss

Bees lost weight much more rapidly during incubation than during wintering (Fig. 6). Because bees wintered for shorter periods required longer incubation periods to emerge, total weight loss diminished with wintering duration (Fig. 6; $F_{(4,21)} = 36.29$; $P < 0.0001$). Total weight loss was 10% in bees wintered for the longest periods (196 and 252 days) compared to 29% in females wintered for 28 days.

3.2.3. Survival and emergence time

Nine of the 10 females wintered for 28 days failed to emerge from the cocoon, and the one that emerged died the day after. In the other treatments, survival was high: 9 of 10 females in the 196-day treatment and 7 of 10 females in the remaining treatments. Emergence time declined with wintering duration (Fig. 6;

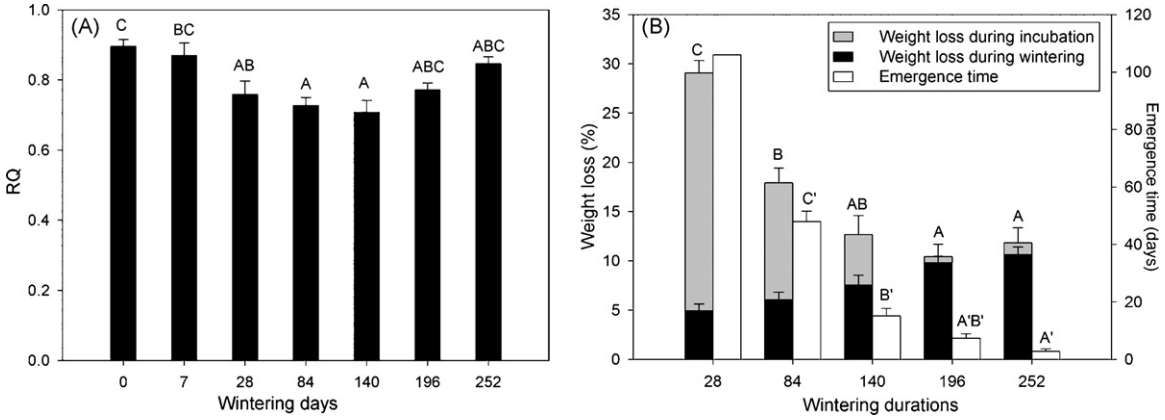

Fig. 6. Mean ± SE respiratory quotient (A) and percentage of weight loss, and emergence time (B) in *O. lignaria* females wintered for 28, 84, 140, 196 and 252 days at 4 °C and incubated at 20 °C.

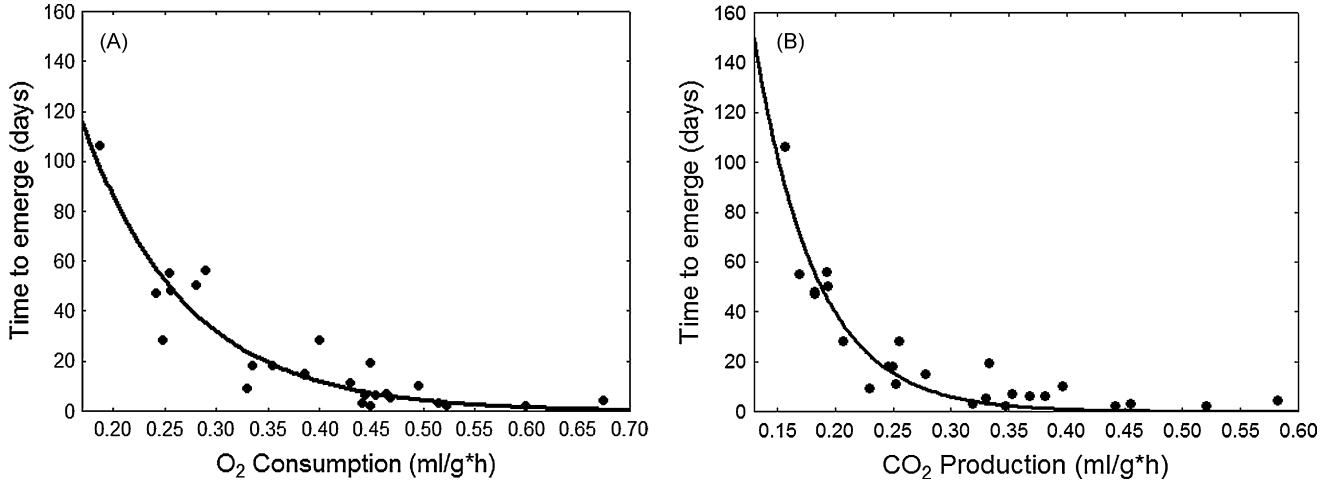

Fig. 7. Relationship of O_2 consumption (A) and CO_2 production (B) measured at the end of wintering with emergence time following incubation at 20 °C (time to emerge = 623.745*exp(−9.902*O_2); R^2 = 0.86; $P < 0.001$; time to emerge = 1709.638*exp(−18.977*CO_2); R^2 = 0.89; $P < 0.001$).

$F_{(3,26)} = 74.153$; $P < 0.001$; 28-day-treatment excluded). Bees wintered for 84 days required 48.00 ± 3.57 days of incubation to emerge versus 2.86 ± 0.83 days in bees wintered for 252 days. The relationship between respiration levels at the end of wintering and emergence time followed a negative exponential curve (Fig. 7).

4. Discussion

4.1. Diapause initiation and maintenance

Respiration rates in newly eclosed *O. lignaria* were ~0.25 ml/g h, and then dropped to ~0.15 ml/g h within 2 weeks, and to ~0.10 ml/g h within 4 weeks (Fig. 5). These results are in agreement with previous studies, which showed a similar pattern in individuals reared either at 22 °C or under natural temperature regimes (Kemp et al., 2004; Sgolastra, 2007). Diapause initiation occurs in individuals reared at constant temperatures in complete darkness, indicating that winter diapause in *O. lignaria* is a fixed component of the ontogenic program, requiring no external cue (obligatory diapause; Tauber et al., 1986; Kostal, 2006).

If not chilled, diapausing *O. lignaria* adults maintain minimum respiration levels (~0.10 ml/g h) until they die. Similar results have been obtained in insects diapausing in different developmental stages, such as larvae of the drosophilid fly *Chymomyza costata* (Kostal et al., 2000), or embryos of the katydid *Eobiana engelhardti* (Higaki and Ando, 2005), showing that a period of cold temperatures is necessary to complete the diapause process. In nature, *O. lignaria* populations reach the adult stage in late summer, shortly before the onset of winter temperatures. Given that diapause initiation (time during which respiration rates drop and reach minimum values) lasts 2–4 weeks, the effective period of diapause maintenance is rather short in this species (Fig. 8).

4.2. Response to chilling and diapause termination

As soon as diapausing adults are chilled, even for periods as short as 7 days, they respond by quickly increasing their respiration rates (measured at 22 °C), showing that the "metabolic brake" (low respiration rates) operating while temperatures were high (pre-winter) has been eased off. Following Kostal (2006), and given that diapause cannot be terminated without chilling, we interpret this response as the beginning of the *process* of diapause termination (Fig. 8). Interestingly, individuals chilled 5 days after adult eclosion (before they have had the time to reach minimum [~0.10 ml/g h] respiration levels) also respond to cold temperature by raising their respiration rate (Sgolastra, 2007). Thus, chilling acts as a synchronizing stimulus among individuals initiating diapause on different dates (Kostal, 2006). The period over which individuals within a population reach adulthood may span for as long as a month (Bosch et al., 2001; Sgolastra, 2007), but the period of emergence lasts only 1–2 weeks (Bosch et al., 2001; Sgolastra, 2007). These results are different from results obtained on another

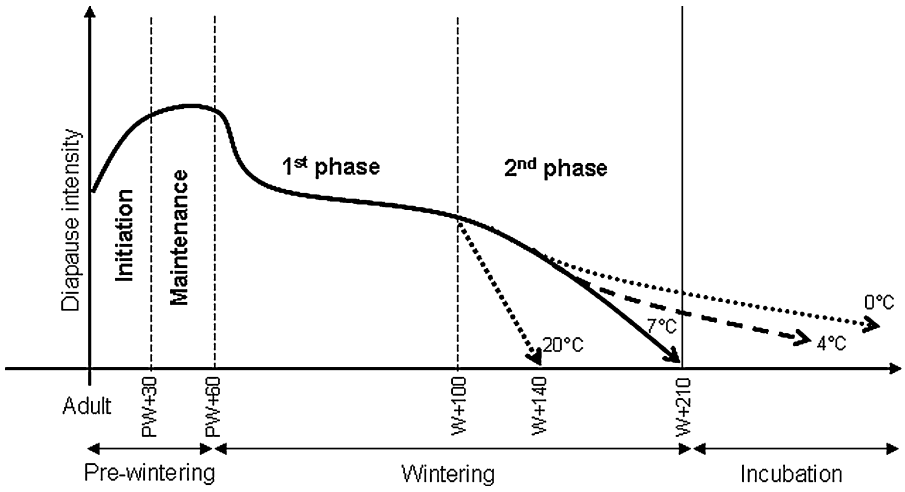

Fig. 8. Schematic depiction (following Kostal, 2006) of diapause intensity during pre-winter (20 °C), winter (0, 4 or 7 °C), and incubation (20 °C) in *O. lignaria* females. Arrow tips indicate emergence out of the cocoon. See Section 4 for interpretation of 1st and 2nd phases.

Megachilid, *Megachile rotundata*. In this species, which enters diapause in summer in the prepupal stage, respiration response remains at 0.1–0.2 ml/g h throughout the autumn and winter, and does not increase until prepupae are exposed to incubation temperatures (Kemp et al., 2004).

Following the initial response to cold temperatures, our respirometry results show two distinct phases (Fig. 8). In the first phase, diapause intensity follows a rapid decrease and then reaches a plateau, during which diapause intensity remains stable or decreases slowly. This first phase lasts ~100 days, and appears to be independent of wintering temperature (within a certain range) (Fig. 2). RQ values, which were as high as ~0.9 during diapause initiation reach minimum levels (~0.7) towards the end of this phase (Figs. 1 and 6), indicating a gradual change in metabolic energy substrates from carbohydrates early on, to lipids (and possibly proteins) in mid-winter (Adedokun and Denlinger, 1985; Hahn and Denlinger, 2007). Also during this first phase, individuals moved from wintering to incubation temperatures (20 °C) respond by lowering their respiration rates (Fig. 5). Instead, in the second phase, diapause intensity decreases exponentially, and this decrease diverges among temperature treatments, being faster at warmer temperatures (Fig. 2). RQ levels increase again, and reach values of ~0.9 when bees approach their emergence time (~150 days in the 7 °C treatment, ~200 days in the 4 °C treatment, April in the outdoors treatment) (Figs. 1, 3 and 6). During this second phase, *O. lignaria* females no longer lower their respiration rates when exposed to 20 °C (Fig. 5).

Following the general diapause model proposed by Kostal (2006), the first phase would correspond to the period of diapause termination, and the second phase to the period of post-diapause quiescence, during which low metabolic rates are maintained exogenously while temperatures are still too cold for morphogenesis (or emergence in *O. lignaria*). According to this interpretation, diapause in *O. lignaria* would be characterized by an early (mid-winter) diapause termination, and a long post-diapause quiescence, in agreement with findings in many other temperate-zone insects (Tauber et al., 1986; Hodek, 2002). This view is supported by results obtained in a previous study (Bosch and Kemp, 2003; see also Bosch and Kemp, 2004) showing reduced winter survival in bees wintered for <90 days, and thus indicating that diapause is clearly not completed at these wintering durations, together with lack of a consistent effect of wintering temperature on emergence time in bees wintered for <90 days. The same study shows that males start emerging without incubation at wintering >150 days at 7 °C, an unequivocal sign that diapause has been completed at this temperature (although males wintered at 0 or 4 °C require incubation temperatures to emerge, even after 270 days of chilling).

The above interpretation, however, does not provide a satisfactory explanation for the relatively long period of incubation required for emergence in bees wintered for 100–150 days. If *O. lignaria* were in post-diapause at such wintering durations, we would expect individuals to respond *immediately to development- or activation-promoting conditions* (Tauber et al., 1986; Kostal, 2006). Yet, *O. lignaria* individuals wintered at 4 °C for 100–150 days and then incubated at 20–22 °C take 7–15 days to emerge, compared to 2–7 days in individuals wintered for 190–240 days (Bosch and Kemp, 2000, 2003; and unpublished data; Bosch et al., 2000; this study). Studies on other *Osmia* species show similar patterns (Taséi, 1973; van der Steen and de Ruijter, 1991; Bosch and Blas, 1994; Bosch and Kemp, 2004; Maeta et al., 2006). Based on these results, ~2 days appears to be the shortest possible emergence time for *O. lignaria* females incubated at ~20 °C. Emergence times of this sort are achieved in individuals whose respiration response have reached levels of ~0.45 ml/g h of CO_2 production and ~0.55 ml/g h of O_2 consumption (Fig. 7). These respiration levels could thus be considered indicators of diapause completion in *O. lignaria*. If incubated when respiration rates have not reached levels close to 0.4 ml/g h (84-day and 140-day treatments), emergence periods are significantly extended, indicating that diapause is still not completed. Unlike males, females do not emerge until exposed to temperatures of ~20 °C, even in fully wintered populations (Bosch and Kemp, 2001; and unpublished data). Females having reached levels of CO_2 production of 0.45 ml/g h would be in post-diapause quiescence until exposed to temperatures eliciting emergence. According to this second interpretation, after ~100 days of wintering, *O. lignaria* would have attained the *potential* to terminate diapause, but diapause completion would not be reached until early spring, and the duration of the termination period would be dependent on temperature (shorter at warmer temperatures). In the field, the respiration response followed a pattern similar to that of treatment 0 °C, and never reached levels close to 0.3 ml/g h until late-March, when temperatures started increasing (Fig. 3). Then, as soon as mean ambient temperatures reached values close to 20 °C in April, the respiration response skyrocketed. The low respiration response recorded until March, and the long emergence periods (15 days) expressed by populations wintered outdoors for insufficiently long periods (117 days) (Bosch et al., 2000), are in agreement with this second interpretation of the second phase.

Future studies should attempt to elucidate which of the two proposed diapause models (early diapause termination followed by a long post-diapause quiescence or late-diapause termination followed by a much abbreviated post-diapause quiescence) is valid for *O. lignaria*. Because physiological processes are usually gradual, our two hypotheses may in fact be two extremes of a continuum. As noted in the introduction, it is often difficult to characterize the transition between the various phases of diapause (Kostal, 2006). This is especially so in *O. lignaria* for two reasons. First, being an obligate diapauser, we cannot compare the response of diapausing and non-diapausing individuals. Second, because diapause occurs in complete darkness, we cannot measure the response to photoperiod throughout wintering. External and internal morphological changes have often been used as indicators of diapause completion (e.g. Kostal et al., 2000; Ragland et al., 2009). Because *O. lignaria* over-winters as a fully formed adult, external morphological changes are not readily apparent, but ovary maturation is far for complete in wintered females (Sgolastra, 2007). Thus, oocyte size could provide a good indicator of morphogenesis resumption in this species. Future studies should also analyze molecular markers (Denlinger, 2002). Patterns of expression during diapause and post-diapause of genes enconding various families of heat-shock proteins have shown promising results in two flies and another Megachilid, the alfalfa leafcutting bee *M. rotundata* (Hayward et al., 2005; Tachibana et al., 2005; Yocum et al., 2005, 2006). In the Heteropteran *Pyrrhocoris apterus*, which winters as an emerged, active adult, levels of transcripts of genes coding for enzymes involved in polyol biosynthesis, were found to closely match diapause intensity and time to oviposition (Kostal et al., 2008).

4.3. Survival and weight loss

Irrespective of the precise timing of diapause termination, exposure to cold temperature is necessary to complete diapause in *O. lignaria*. Non-chilled bees, as well as bees chilled for very short periods, respond to warm temperatures by lowering their respiration, and thus never reach levels eliciting emergence (Figs. 1 and 5). However, even while maintaining low respiration rates, diapausing individuals kept at high temperatures incur in high metabolic cost, resulting in extensive fat body depletion, loss of vigour and increased mortality (Bosch and Kemp, 2004; Sgolastra, 2007; Bosch et al., 2008). Of course, these losses would be much greater did bees not keep their metabolic rates low under these conditions. At any rate, none of the bees exposed to the no-wintering treatment survived. In insects diapausing as feeding stages, increased metabolic activity during periods of warm weather in the initiation phase is compensated by feeding and building up of energy reserves (Tauber et al., 1986; Kostal, 2006; Hahn and Denlinger, 2007), a possibility not available to pre-wintering *Osmia* adults. Both in *O. lignaria* and in *O. cornuta*, weight loss rates are much higher during pre-wintering (0.2–0.4 mg/day) than during wintering (0.05–0.09 mg/day) (Bosch and Kemp, 2003, 2004; Kemp et al., 2004).

Only one of the females exposed to a very short winter (28 days) survived to emergence. This result is consistent with other *Osmia* studies in which survival of bees wintered for $\leq$30 days, ranged between 0 and 40% (Bosch and Kemp, 2003, 2004; Maeta et al., 2006). In addition, those individuals that manage to emerge following short wintering periods are weak and have short post-emergence longevity (Bosch and Kemp, 2003, 2004). Conversely, in the bumblebee *B. terrestris* survival was not negatively affected by short wintering periods (1 month; Beekman et al., 1998). *O. lignaria* incubated following a short wintering period respond by rapidly lowering their metabolism, but nonetheless loose weight rapidly at a rate similar to that expressed during pre-wintering (Fig. 6). Because these bees require long incubation periods to emerge (Fig. 6), the end result is a very high weight loss. Metabolic depression is a common response to environmental stress, but is usually accompanied by a decrease in fitness (Parsons, 1996; Chown and Gaston, 1999).

In addition to wintering duration, winter temperature also affects *O. lignaria* fitness. Bees exposed to milder winter temperatures emerge sooner, but express increased weight loss and fat body depletion, resulting in decreased survival and poor vigour at emergence (Bosch and Kemp, 2003). Shorter diapause duration at warmer temperatures has been attributed to the greater metabolic rate and increased catabolism of nutrient resources (Hahn and Denlinger, 2007). Studies on the ladybird beetle *Coleomegilla maculata* and the gall fly *Eurosta solidaginis* have also shown reduced survival and vigour in individuals wintered at warm temperatures (Jean et al., 1990; Irwin and Lee, 2000). Conversely, wintering temperature did not affect winter survival in *B. terrestris* (Beekman et al., 1998). In nature, earlier emergence in years with milder winters could serve as a mechanism to synchronize *O. lignaria* emergence with advanced blooming time of its vernal host plants. However, increased body weight loss and fat body depletion during mild winters may compromise post-diapause performance. A trade-off between diapause and reproductive success has been reported in several insects (Bradshaw et al., 1998; Irwin and Lee, 2000; Ellers and van Alphen, 2002; Musolin and Numata, 2003), but losses incurred during suboptimal winters may be off-set in species whose individuals feed before reproducing, as is the case in *O. lignaria* (Peferoen et al., 1981; Jansson et al., 1989; Ishihara and Shimada, 1995).

Management methods have been developed to use *O. lignaria* and other *Osmia* species for orchard pollination (Bosch and Kemp, 2002). By improving our understanding of the ecophysiological processes underlying winter diapause, our results should help to improve temperature regimes currently used to rear *Osmia* populations. In addition, the use of respiration rates as an indicator of emergence time may help to improve synchronization between bee emergence and blooming of the target crop.

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References

Adedokun, T.A., Denlinger, D.L., 1985. Metabolic reserves associated with pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. Journal of Insect Physiology 31, 229–233.

Andrewartha, H.G., 1952. Diapause in relation to the ecology of insects. Biological Reviews 27, 50–107.

Beekman, M., van Stratum, P., Lingeman, R., 1998. Diapause survival and post-diapause performance in bumblebee queens (*Bombus terrestris*). Entomologia Experimentalis et Applicata 89, 207–214.

Bosch, J., Blas, M., 1994. Effect of over-wintering and incubation temperatures on adult emergence in *Osmia cornuta* Latr (Hymenoptera, Megachilidae). Apidologie 25, 265–277.

Bosch, J., Kemp, W.P., 2000. Development and emergence of the orchard pollinator, *Osmia lignaria* (Hymenoptera: Megachilidae). Environmental Entomology 29, 8–13.

Bosch, J., Kemp, W.P., 2001. How to Manage the Blue Orchard Bee as an Orchard Pollinator. Sustainable Agriculture Network, Beltsville, Maryland.

Bosch, J., Kemp, W.P., 2002. Developing and establishing bee species as crop pollinators: the example of *Osmia* spp. (Hymenoptera: Megachilidae) and fruit trees. Bulletin of Entomological Research 92, 3–16.

Bosch, J., Kemp, W.P., 2003. Effect of wintering duration and temperature on survival and emergence time in males of the orchard pollinator *Osmia lignaria* (Hymenoptera: Megachilidae). Environmental Entomology 32, 711–716.

Bosch, J., Kemp, W.P., 2004. Effect of pre-wintering and wintering temperature regimes on weight loss, survival, and emergence time in the mason bee *Osmia cornuta* (Hymenptera: Megachilidae). Apidologie 35, 469–479.

Bosch, J., Kemp, W.P., Peterson, S.S., 2000. Management of *Osmia lignaria* (Hymenoptera: Megachilidae) populations for almond pollination: methods to advance bee emergence. Environmental Entomology 29, 874–883.

Bosch, J., Maeta, Y., Rust, R.W., 2001. A phylogenetic analysis of nesting behavior in the genus *Osmia* (Hymenoptera: Megachilidae). Annals of the Entomological Society of America 94, 617–627.

Bosch, J., Sgolastra, F., Kemp, W.P., 2008. Life cycle ecophysiology of *Osmia* mason bees. In: James, R.R., Pitts-Singer, T.L. (Eds.), Bee Pollination in Agricultural Ecosystems. Oxford University Press, pp. 83–104.

Bradshaw, W.E., Armbruster, P.A., Holzapfel, C.M., 1998. Fitness consequences of hibernal diapause in the pitcher-plant mosquito *Wyeomyia smithii*. Ecology 79, 1458–1462.

Chown, S.L., Gaston, K.J., 1999. Exploring links between physiology and ecology at macro-scales: the role of respirometry in metabolism in insects. Biology Reviews 74, 87–120.

Danks, H.V., 1987. Insect dormancy: an ecological perspective. Biological Survey of Canada, Monograph Series No. 1, Ottawa.

Denlinger, D.L., 2002. Regulation of diapause. Annual Review of Entomology 47, 93–122.

Ellers, J., van Alphen, J.J.M., 2002. A trade-off between diapause duration and fitness in female parasitoids. Ecological Entomology 27, 279–284.

Gauld, I.D., Bolton, B., 1988. The Hymenoptera. British Museum (Natural History) and Oxford University Press, London and Oxford, p. 332.

Gray, D.R., Ravlin, F.W., Régnière, J., Logan, J.A., 1995. Further advances toward a model of gypsy moth (*Lymantria dispar* (L.)) egg phenology: respiration rates and thermal responsiveness during diapause and age-dependent development rates in postdiapause. Journal of Insect Physiology 41, 247–256.

Hahn, D.A., Denlinger, D.L., 2007. Meeting the energetic demands of insect diapause: nutrient storage and utilization. Journal of Insect Physiology 53, 760–773.

Hayward, S.A.L., Pavlides, S.C., Tammariello, S.P., Rinehart, J.P., Denlinger, D.L., 2005. Temporal expression patterns of diapause associated genes in flesh fly pupae from the onset of diapause through post-diapause quiescence. Journal of Insect Physiology 51, 631–640.

Higaki, M., Ando, Y., 2005. Effects of temperature during chilling and pre-chilling periods on diapause and post-diapause development in a katydid, *Eobiana engelhardti subtropica*. Journal of Insect Physiology 51, 709–716.

Hodek, I., 2002. Controversial aspects of diapause development. European Journal of Entomology 99, 163–173.

Hodek, I., Hodková, M., 1988. Multiple role of temperature during insect diapause: a review. Entomologia Experimentalis et Applicata 49, 153–165.

Hodek, I., Honek, A., 1996. Ecology of Coccinellidae. Series Entomologica, vol. 54. Kluwer Academic, Dordrecht, Netherlands.

Hodková, M., Hodek, I., 1989. Role of feeding, photoperiod and temperature in the regulation of reproduction by the neuroendocrine system in *Pyrrhocoris apterus*. In: Tonner, M., Soldan, T., Bennetova, B. (Eds.), Regulation of Insect Reproduction IV. Academia, Praha, pp. 377–384.

Irwin, T.J., Lee, R.E., 2000. Mild winter temperatures reduce survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae). Journal of Insect Physiology 46, 655–661.

Ishihara, M., Shimada, M., 1995. Trade-off in allocation of metabolic reserves: effects of diapause on egg production and adult longevity in a multivoltine bruchid *Kytorhinus sharpianus*. Functional Ecology 9, 618–624.

Jansson, R.K., Zitzman Jr., A.E., Lashomb, J.H., 1989. Effects of food plant and diapause on adult survival and fecundity of Colorado potato beetle (Coleoptera: Chrysomelidae). Environmental Entomology 18, 291–297.

Jean, C., Coderre, D., Tourneur, J.-C., 1990. Effects of temperature and substrate on survival and lipid consumption of hibernating *Coleomegilla maculata lengi* (Coleoptera: Coccinellidae). Environmental Entomology 19, 1657–1662.

Kemp, W.P., Bosch, J., 2005. Effect of temperature on *Osmia lignaria* (Hymenoptera: Megachilidae) prepupa-adult development, survival, and emergence. Journal of Economic Entomology 98, 1917–1923.

Kemp, W.P., Bosch, J., Dennis, B., 2004. Oxygen consumption during the life cycle of the prepupa-wintering bee *Megachile rotundata* (F.) and the adult-wintering bee *Osmia lignaria* Say (Hymenoptera: Megachilidae). Annals of the Entomological Society of America 97, 161–170.

Kostal, V., 2006. Eco-physiological phases of insect diapause. Journal of Insect Physiology 52, 113–127.

Kostal, V., Shimada, K., Hayakawa, Y., 2000. Induction and development of winter larval diapause in a drosophilid fly *Chymomyza costata*. Journal of Insect Physiology 46, 417–428.

Kostal, V., Tollarová, M., Doležel, D., 2008. Dynamism in physiology and gene transcription during reproductive diapause in a heteropteran bug, *Pyrrhocoris apterus*. Journal of Insect Physiology 54, 77–88.

Maeta, Y., Nakanishi, K., Fujii, K., Kitamura, K., 2006. Exploitation of systems to use a univoltine Japanese mason bee *Osmia cornifrons* (Radoszkowski), throughout the year for pollination of greenhouse crops (Hymenoptera: Megachilidae). Chugoku Kontyu 20, 1–17.

Musolin, D.L., Numata, H., 2003. Timing of diapause induction and its life-history consequences in *Nezara viridula*: is it costly to expand the distribution range? Ecological Entomology 28, 694–703.

Parsons, P.A., 1996. Stress, resources, energy balances, and evolutionary change. Evolutionary Biology 29, 39–72.

Peferoen, M., Huybrechts, R., De Loof, A., 1981. Longevity and fecundity in the Colorado potato beetle *Leptinotarsa decemlineata*. Entomologia Experimentalis et Applicata 29, 321–329.

Ragland, G.J., Fuller, J., Feder, J.L., Hahn, D.A., 2009. Biphasic metabolic rate trajectory of pupal diapause termination and post-diapause development in a tephritid fly. Journal of Insect Physiology 55, 344–350.

Sawyer, A.J., Tauber, M.J., Tauber, C.A., Ruberson, J.R., 1993. Gypsy moth (Lepidoptera: Lymantriidae) egg development: a simulation analysis of laboratory and field data. Ecological Modelling 66, 121–155.

Sgolastra, F., 2007. Ecofisiologia del ciclo biologico di *Osmia lignaria* (Hymenoptera: Megachilidea). Unpublished doctoral dissertation, Università di Bologna.

Singtripop, T., Saeangsakda, M., Tatun, N., Kaneko, Y., Sakurai, S., 2007. Correlation of oxygen consumption, cytochrome c oxidase, and cytochrome c oxidase subunit I gene expression in the termination of larval diapause in the bamboo borer, *Omphisa fuscidentalis*. Journal of Insect Physiology 53, 933–939.

Stephen, W.P, Undurraga, J.M., 1976. X-radiography, an analytical tool in population studies of the leafcutter bee, *Megachile pacifica*. Journal of Apicultural Research 15, 81–87.

Stephen, W.P., Bohart, G.E., Torchio, P.F., 1969. The Biology and External Morphology of Bees. Agricultural Experiment Station, Oregon State University, Corvallis, p. 140.

Tachibana, S.I., Numata, H., Goto, S.G., 2005. Gene expression of heat-shock proteins (Hsp23, Hsp70 and Hsp90) during and after larval diapause in the blow fly *Lucilia sericata*. Journal of Insect Physiology 51, 641–647.

Taséi, J.N., 1973. Observations sur le development d'*Osmia cornuta* Latr. et *Osmia rufa* L. (Hymenoptera, Megachilidae). Apidologie 3, 295–315.

Tauber, M.J., Tauber, C.A., Masaki, S., 1986. Seasonal Adaptations of Insects. Oxford University Press, Oxford, UK.

Torchio, P.F., 1985. Field experiments with the pollinator species, *Osmia lignaria* propinqua Cresson in apple orchards: V. (1979–1980), methods of introducing bees, nesting success, seed counts, fruit yields (Hymenoptera: Megachilidae). Journal of the Kansas Entomological Society 58, 448–464.

Torchio, P.F, 1989. In-nest biologies and immature development of three *Osmia* species (Hymenoptera: Megachilidae). Annals of the Entomological Society of America 82, 599–615.

van der Steen, J., de Ruijter, A., 1991. The management of *Osmia rufa* L. for pollination of seed crops in greenhouses. Proceedings of Experimental and Applied Entomology 2, 137–141.

Yaginuma, T., Yamashita, O., 1999. Oxygen consumption in relation to sorbitol utilization at the termination of diapause in eggs of the silkworm, *Bombyx mori*. Journal of Insect Physiology 45, 621–627.

Yocum, G.D., Kemp, W.P., Bosch, J., Knoblett, J.N., 2005. Temporal variation in overwintering gene expression and respiration in the solitary bee *Megachile rotundata*. Journal of Insect Physiology 51, 621–629.

Yocum, G.D., Kemp, W.P., Bosch, J., Knoblett, J.N., 2006. Thermal history influences diapause development in the solitary bee *Megachile rotundata*. Journal of Insect Physiology 52, 1113–1120.